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INTRODUCTION

The **subject** of this project is “colorectal cancer” and the focus area is “inflammatory response in the development of colorectal cancer”. Inflammatory bowel disease (IBD) is associated with the development of colorectal cancer; however, little is known about the mechanisms responsible for the progression of IBD to malignancy. The **purpose** of the work is to address the hypothesis that aberrant expression of the mucin 1 (MUC1) oncoprotein by chronic intestinal inflammation contributes to the development of colorectal cancers that are in turn dependent on MUC1 for the malignant phenotype. The **scope** of the research is to define the role of MUC1 in the growth and survival of human colon cancer cells growing in vitro and as tumor xenografts in nude mice and to develop a MUC1⁺/IL-10^{-/-} model of colitis to determine whether disrupting MUC1 function affects the inflammatory response and colon tumorigenesis.

BODY

Task 3 (Months 1-12): To define the mechanisms associated with inhibiting MUC1-C that block growth and survival of human colon cancer cells growing in vitro and as xenografts in nude mice.

MUC1 is a heterodimer that consists of (i) an N-terminal mucin subunit (MUC1-N), and (ii) a C-terminal transmembrane subunit (MUC1-C) that functions as an oncoprotein (1-3). Overexpression of MUC1-C, as found in colorectal cancer cells, is sufficient to induce anchorage-independent growth and tumorigenicity (1, 3). The MUC1-C subunit consists of a 72 aa cytoplasmic domain with a CQC motif that is necessary for MUC1-C dimerization and oncogenesis (1, 3). In this regard, we have developed a cell-penetrating peptide drug, designated GO-203 (Fig. 1A), that targets the MUC1-C CQC motif and thereby blocks its dimerization and oncogenic function (4-6). As a control, we have synthesized CP-3, a cell-penetrating peptide in which the CQC motif has been altered to AQA (Fig. 1A), that does not bind to MUC1-C and thereby has no effect on MUC1-C function (4-6).

MUC1-positive SK-CO-1 (Fig. 1B), COLO-205 (Fig. 1C) and COLO-320 (Fig. 1D) colorectal cancer cells were left untreated (Control) and treated with 2.5 μ M GO-203 or CP-3 for the indicated times. Treatment with GO-203 was associated with inhibition of growth and loss of survival (late apoptosis/necrosis) (Figs. 1B-D). By contrast, the control CP-3 peptide had little if any effect (Figs. 1B-D). In addition, treatment of MUC1-negative HCT116 colon cancer cells with GO-203 was ineffective in inhibiting growth (Fig. 1E). These findings demonstrate that human colorectal cancer cells that express MUC1 are dependent on MUC1-C for their growth and survival.

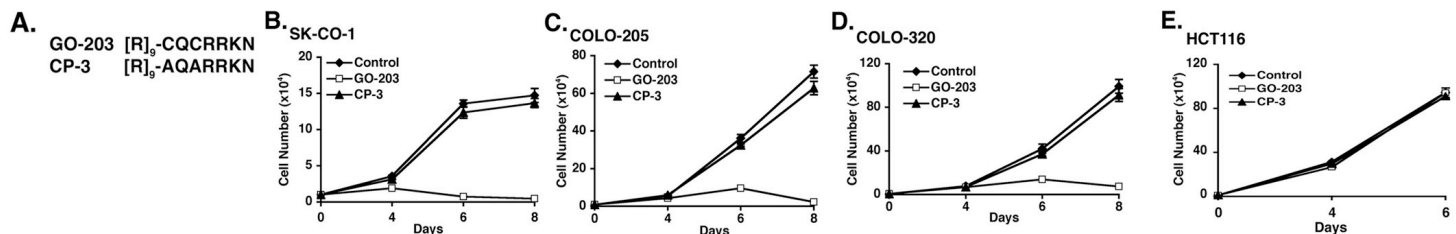


Figure 1. Targeting MUC1-C with GO-203 in colorectal cancer cell lines growing in vitro. A. Amino acid sequences of the GO-203 and CP-3 cell-penetrating peptides. B-E. SK-CO-1 (B; MUC1-positive), COLO-205 (C; MUC1-positive), COLO-320 (D; MUC1-positive), and HCT116 (E; MUC1-negative) were left untreated (Control) and treated with 2.5 μ M GO-203 or CP-3 for the indicated times. Viable cell number was determined by trypan blue staining.

To extend these results obtained from in vitro experiments, we established subcutaneous COLO-205 colon tumor xenografts (~90 mm³) in the flanks of nude mice. Groups of 8 mice each were treated intraperitoneally (ip) with PBS (Control), 18 mg/kg GO-203, or 18 mg/kg CP-3 each day for 28 days (Fig. 2A). Compared to the control group, growth of the COLO-205 tumors was inhibited in the GO-203-treated mice (Fig. 2A). Moreover, these tumors regressed completely by the end of treatment (day 28) and there was no evidence for regrowth by day 180 (Fig. 2A). In contrast to the anti-tumor activity of GO-203, treatment with CP-3 had no effect on COLO-205 tumor growth (Fig. 2A). Importantly, GO-203 was not associated with loss of body weight or other apparent toxicities. Studies were also performed on established subcutaneous SK-CO-1 tumor xenografts (~90 mm³) to explore other GO-203 dose-schedules (Fig. 2B). Groups of 8 mice each were

treated with PBS (Control), 6 mg/kg GO-203 each day for 5 days intravenously (iv), or 3 mg/kg GO-203 each day for 5 days iv each week for two weeks. The results demonstrate that, as compared to the control group, growth of tumors in the mice treated with GO-203 was inhibited with both dose-schedules (Fig. 2B). These findings indicate that GO-203 is effective in inhibiting growth and survival of MUC1-positive colorectal cancer cells in mouse xenograft models. The findings also support a GO-203 dose-response effect with regression of tumors at higher doses (18 mg/kg) administered for longer periods (28 days).

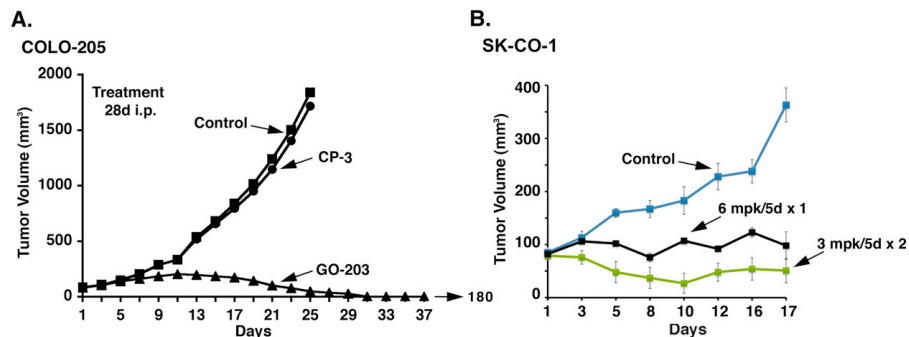


Figure 2. GO-203 is effective against COLO-205 and SK-CO-1 colon cancer xenografts growing in nude mice. A and B. BALB/c nu/nu mice were injected subcutaneously in the flank with 1×10^7 COLO-205 (A) or SK-CO-1 (B) cells. The mice were pair matched when the tumors were $\sim 100 \text{ mm}^3$. A. Treatment groups consisted of 8 mice injected intraperitoneally (ip) with PBS (vehicle control), 18 mg/kg GO-203 or 18 mg/kg CP-3 each day for 28 days. B. Treatment groups consisted of 8 mice injected iv with PBS, 6 mg/kg GO-203 each day for 5 days, or 3 mg/kg GO-203 each day for 5 days each week for 2 weeks. Tumor measurements were performed every 4 days and mice were weighed twice weekly. There was no weight loss in any of the groups. The results are expressed as the mean tumor volume with a SE of less than 20%.

The MUC1-C subunit can interact with different effectors, such as NF- κ B, STAT3 and Wnt/ β -catenin, and contribute to the activation of their respective pathways (7-11). Analysis of colorectal cancer cells treated with GO-203 demonstrated that targeting MUC1-C has little if any effect on the constitutive activation of the STAT3 and Wnt/ β -catenin pathways. By contrast, GO-203 treatment was partially effective in suppressing NF- κ B signaling and thereby a pathway linked to inflammation. K-RAS is of importance to colon cancer cell growth and survival (12), and MUC1-C contributes to the K-RAS pathway through interactions with GRB2/SOS (1, 3) (Fig. 3A). In this context, we found that GO-203 treatment of COLO-205 and other colorectal cancer cells is associated with downregulation of MEK \rightarrow ERK signaling (Fig. 3B). These findings thus provided a potential mechanism for the effects of GO-203 treatment on colorectal cancer cell growth and survival.

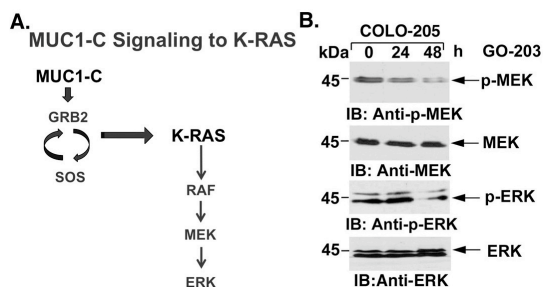


Figure 3. Targeting MUC1-C with GO-203 results in downregulation of the RAS \rightarrow RAF \rightarrow MEK \rightarrow ERK pathway. A. Schema. The MUC1-C cytoplasmic domain interacts with GRB2/SOS (1), linking MUC1-C to activation of the RAS pathway. B. COLO-205 cells were treated with 2.5 μ M GO-203 for the indicated times. Lysates were immunoblotted with the indicated antibodies.

To provide further evidence for involvement of MUC1-C in RAS \rightarrow MEK \rightarrow ERK signaling, we investigated the effects of combining GO-203 with the MEK inhibitor, AS703026. Treatment of SK-CO-1 cells with a low inactive GO-203 dose or a partially active concentration of AS703026 demonstrated that the combination is highly effective in inhibiting colony formation (Fig. 4A, upper panel). Isobologram analysis of different concentrations of GO-203 and AS703026 confirmed a high degree of synergism with a Combination Index (CI) of less than 0.1 (Fig. 4A, lower panels). Similar results were obtained when GO-203 was combined with the MEK inhibitor, GSK1120212 (Fig. 4B, upper panel), and demonstrated a CI of 0.1 (Fig. 4B, lower panels). These findings indicate that targeting MUC1-C with GO-203 in colorectal cancer cells is an effective approach for suppressing the RAS \rightarrow MEK \rightarrow ERK pathway and thereby inhibiting their growth and survival.

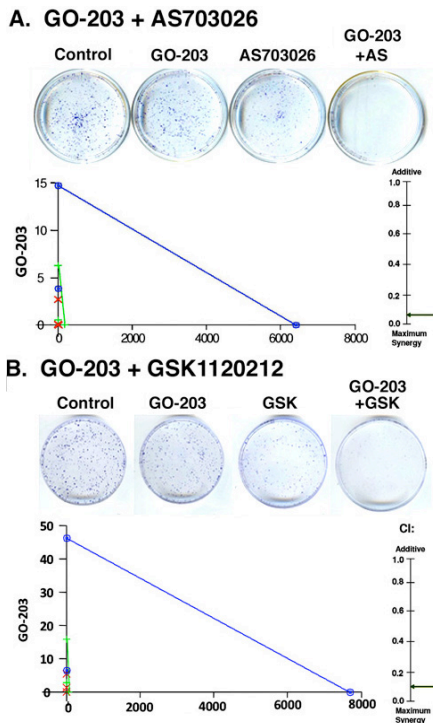


Figure 4. Targeting MUC1-C is synergistic with MEK inhibitors in suppressing colorectal cancer cell survival. A. SK-CO-1 cells were treated with 0.6 μ M GO-203, 1.2 μ M AS703026 or the combination of both the agents for 48 h. GO-203 was added every 24 h. The cells were then incubated in the absence of drugs for 12 days and stained with crystal violet (upper panel). Isobologram was generated by exposing the cells to fixed IC_{50} ratios of GO-203, AS703026 and the GO-203/AS703026 combination. The multiple effect-level isobologram analysis is shown for ED 90 (\odot), ED 75 ($+$) and ED 50 (\times) with the right side panel indicating the CI index for the combination (lower panels). B. SK-CO-1 cells were exposed to 1.22 μ M GO-203, 0.0096 μ M GSK1120212 or the combination of both the agents for 48 h. GO-203 was added every 24 h. The cells were then incubated in the absence of drugs for 12 days and stained with crystal violet (upper panel). Isobologram was generated by exposing the cells to fixed IC_{50} ratios of GO-203, GSK1120212 and the GO-203/GSK1120212 combination. The multiple effect-level isobologram analysis is shown for the ED 90 (\odot), ED 75 ($+$) and ED 50 (\times) with the right side panel indicating the CI index for the combination (lower panels).

These findings collectively indicate that MUC1-C is of importance to the growth and survival of human colorectal cancer cells growing in vitro and as tumor xenografts.

Tasks 1 and 2 (Months 6-12): To develop a MUC1⁺/IL-10^{-/-} mouse model of colitis for (i) determining if disruption of MUC1-C function with GO-203 affects the inflammatory response and colon tumorigenesis, and (ii) assessing the effects of inhibiting MUC1-C function on activation of signaling pathways that have been linked to inflammation and tumorigenesis.

To develop a mouse model of colitis and tumorigenesis, we first crossed MUC1^{+/+} (here MUC1 represents human MUC1) mice with IL-10^{-/-} mice (obtained from Jackson Laboratories) (Fig. 6A). Genotyping was used for identification of F1 MUC1^{+/+}/IL-10^{-/-} mice (Fig. 7B), which were then bred with IL-10^{-/-} mice (Fig. 7A). Results from genotyping the F2 mice demonstrated MUC1^{+/+}/IL-10^{-/-} (#1,2,5,6,7,8,10, and 12), MUC1^{+/+}/IL-10^{+/+} (#3,4, and 14), and IL-10^{+/+} (#9,11,13 and 15) pups (Fig. 6B).

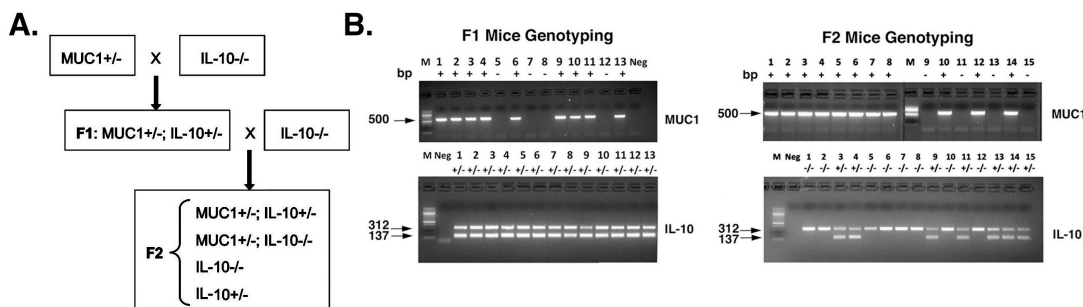


Figure 6. Generation of a MUC1⁺/IL-10^{-/-} mouse model of colitis. A. Schema for the breeding strategy of F1 and F2 mice. B. Genotyping of the F1 and F2 pups by PCR for MUC1 and IL-10.

MUC1^{+/+}/IL-10^{-/-} mice have been monitoring for weight loss and rectum prolapse twice a week along with wild-type, MUC1^{+/+} and IL-10^{-/-} mice. Once mice show weight loss or rectum prolapse (especially MUC1^{+/+}/IL-10^{-/-} or IL-10^{-/-} mice), colon and small intestine tissues will be harvested for immunohistochemistry study. We expect that MUC1^{+/+}/IL-10^{-/-} mice will develop colitis at earlier age than IL-10^{-/-} mice and also develop colon cancer at late stage of inflammation. Then we will treat IL-10^{-/-} mice with GO-203 peptide to check if disruption of MUC1 function slows down or even blocks colitis development in IL-10^{-/-} mice. Mechanism studies will also be done on the function of MUC1 in colitis and colon tumor development.

KEY RESEARCH ACCOMPLISHMENTS

The key research accomplishments emanating from this ongoing research are that: (1) MUC1-positive human colon cancer cell lines are dependent on the MUC1-C oncoprotein for their growth and survival in vitro and in xenograft models; (2) targeting MUC1-C with GO-203 is associated with suppression of the RAS→MEK→ERK pathway, which has been shown to be of importance for colon tumorigenesis; and (3) preliminary results further indicate that targeting MUC1-C with GO-203 is associated with inhibition of NF-κB signaling and may represent a potential link among inflammation, MUC1-C and development of colon tumors.

REPORTABLE OUTCOMES

There are no reportable outcomes from this first year of research funding.

CONCLUSION

Our results to date from studies of human colorectal cancer cells growing in vitro and in vivo provide the first evidence that MUC1-C is a potential target for the treatment of this disease.

REFERENCES

1. Kufe, D, *Mucins in cancer: function, prognosis and therapy*. Nature Reviews Cancer, 2009. 9:874-85.
2. Kufe, D, *Oncogenic function of the MUC1 receptor subunit in gene regulation*. Oncogene, 2010. 29:5663-6.
3. Kufe, D, *MUC1-C oncoprotein as a target in breast cancer: activation of signaling pathways and therapeutic approaches*. Oncogene 2013. 32:1073-81.
4. Raina, D, Ahmad R, Joshi M, Yin L, Wu Z, Kawano T, Vasir B, Avigan D, Kharbanda S, and Kufe D, *Direct targeting of the MUC1 oncoprotein blocks survival and tumorigenicity of human breast carcinoma cells*. Cancer Res, 2009. 69:5133–41.
5. Raina, D, Kosugi M, Ahmad R, Panchamoorthy G, Rajabi H, Alam M, Shimamura T, Shapiro G, Supko J, Kharbanda S, and Kufe D, *Dependence on the MUC1-C oncoprotein in non-small cell lung cancer cells*. Mol Cancer Therapeutics, 2011. 10:806-16.
6. Raina, D, Ahmad R, Rajabi H, Panchamoorthy G, Kharbanda S, and Kufe D, *Targeting cysteine-mediated dimerization of the MUC1-C oncoprotein in human cancer cells*. Int J Oncol, 2012. 40:1643-9.
7. Ahmad, R, Raina D, Trivedi V, Ren J, Rajabi H, Kharbanda S, and Kufe D, *MUC1 oncoprotein activates the IκB kinase β complex and constitutive NF-κB signaling*. Nat Cell Biol, 2007. 9:1419-27.
8. Ahmad, R, Raina D, Joshi MD, Kawano T, Kharbanda S, and Kufe D, *MUC1-C oncoprotein functions as a direct activator of the NF-κB p65 transcription factor*. Cancer Res, 2009. 69:7013-21.
9. Ahmad, R, Rajabi H, Kosugi M, Joshi M, Alam M, Vasir B, Kawano T, Kharbanda S, and Kufe D, *MUC1-C oncoprotein promotes STAT3 activation in an auto-inductive regulatory loop*. Science Signaling, 2011. 4:ra9.
10. Huang, L, Chen D, Liu D, Yin L, Kharbanda S, and Kufe D, *MUC1 oncoprotein blocks GSK3β-mediated phosphorylation and degradation of β-catenin*. Cancer Res, 2005. 65:10413-22.
11. Rajabi, H, Ahmad R, Jin C, Kosugi M, Alam M, Joshi M, and Kufe D, *MUC1-C oncoprotein induces TCF7L2 activation and promotes cyclin D1 expression in human breast cancer cells*. J Biol Chem, 2012. 287:10703-13.
12. Pylayeva-Gupta, Y, Grabocka E, and Bar-Sagi D, *RAS oncogenes: weaving a tumorigenic web*. Nat Rev Cancer, 2011. 11:761-74.

APPENDICES

None.

SUPPORTING DATA

The figures and their legends have been incorporated into the above text.